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14. ABSTRACT

Androgen signaling via its receptor, AR, is a key therapeutic target in prostate cancer. Despite new treatments, resistance is a huge problem. Our goal is to inhibit AR target genes that drive cancer growth but not those for normal cell survival. Our hypothesis is that these sets of genes differ in androgen response elements (AREs), with genes driving proliferation relying on consensus inverted repeats (cARE) and genes promoting differentiation relying on selective direct repeats or half-sites (sAREs). To identify compounds that may interact with AR to affect DNA recognition, we developed a high-throughput screen for compounds eliciting different AR activity on cARE vs. sARE reporters. Over 10,000 compounds were tested. Doxorubicin proved best at differentially affecting AR-dependent gene expression in LNCaP cells, by interacting with DNA rather than directly with AR. Doxorubicin is known to elicit DNA damage response, a pathway also influenced by AR. We used protein-DNA interaction assays to show the differential effect of dox on AR binding *in vitro*, and have extended this to show selectivity of AR binding *in vivo* by chromatin immunoprecipitation (ChIP) studies. Further, we have extended our findings of differential gene action by dox to novel regulatory interactions between AR and known tumor suppressors and oncogenes.

15. SUBJECT TERMS
androgen receptor, prostate cancer, antiandrogens, high-throughput screen, differential promoter activation, selective response elements

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1. INTRODUCTION: Prostate cancer (PCa) initially depends on androgens acting via the androgen receptor (AR), and thus blocking steroid synthesis and AR function slows disease. Yet even with potent new drugs, resistance arises and AR regains control. An innovative approach to deter resistance is to identify selective AR modulators (SARMs) that prevent expression of genes promoting cancer growth but enable expression of genes for differentiation. Such genes may differ in androgen response elements (AREs) and/or cofactor binding sites. In support of this idea, genes involved in differentiation appear to rely on a more selective ARE (sARE) than the consensus ARE (cARE) that is shared with other steroid receptors. The consensus cARE, an inverted repeat of AGAACA half sites with a 3 nt spacer, is recognized by dimers of either AR or glucocorticoid receptor (GR), while the more flexible sARE is an imperfect direct repeat or a half-site that can be bound by one AR monomer within the requisite dimer (Fig. 1).

To identify SARMs that elicit differential gene expression, we developed a high-throughput multiplexed promoter-dependent compound screen, aided by the University of Michigan Center for Chemical Genomics (CCG). Differential AR activation in transfected cells was assessed using fluorescent reporter genes driven by multimerized cARE or sARE promoters. This strategy targets AR regardless of hormone presence or receptor variation (e.g., splice forms), and may identify compounds contacting any AR domain or interacting with other components of the AR signaling pathway. One hit, doxorubicin (dox), selectively inhibited cARE-driven fluorescence and was further validated in luciferase assays in prostate cells. We hypothesized that selective interactions of dox with different DNA sequences could differentially interfere with AR binding and thus differentially affect AR target gene activation. We found that AR-cARE binding *in vitro* is more sensitive to disruption by dox than AR-sARE binding. Moreover, in chromatin dox inhibits AR recruitment to cAREs but promotes AR recruitment to sAREs. In LNCaP cells, dox treatment represses cARE-driven but, at low concentrations, increases sARE-driven target gene expression, including genes associated with differentiation or growth arrest. This data provides proof of principle that compounds can differentially regulate AR target genes via selective interaction with distinct cis regulatory elements to drive different cell behaviors.

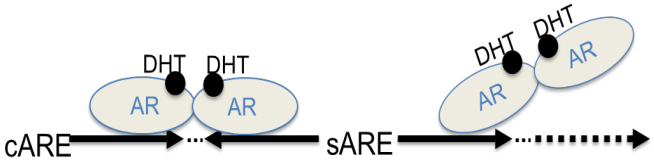

Fig. 1. AR dimers bind distinctly to cAREs vs. sAREs. The cARE inverted repeat is recognized by either AR or GR. The sARE half-site or direct repeat is only recognized by AR (*1*). AR head-to-head dimers bind both half sites of a cARE but only one half site of a sARE.

2. KEYWORDS: androgen receptor, prostate cancer, antiandrogens, high-throughput screen, selective androgen receptor modulator, doxorubicin

3. ACCOMPLISHMENTS: The major goals stated in the original Statement of Work were:

Task 1. Validate primary screen hits as modulators of AR gene regulation and define mechanisms.
Task 2. Perform an optimized and larger screen for additional diverse SARMS.
Task 3. Test antitumor efficacy of selective modulators *in vivo*.

Progress Report Revision. In the first year of this project significant progress was made, but difficulties became apparent in the second year, exacerbated by an accident that kept the PI out of the laboratory for two months, with ongoing rehabilitation. A strategy viewed as beneficial in obtaining the grant (noted by a reviewer as overambitious) was to pursue Task 1 and Task 2 simultaneously. Task 2 however failed to produce additional promising hits, despite optimization of the screening methodology, as we described in the Progress Report for 2015. One hit dubbed Cpd05 was similar to doxorubicin in some aspects of selectivity, but failed to increase AR induction of SGK1, which is the hallmark gene whose activation depends on an sARE. Furthermore, Cpd05 had poor solubility and extensive chemical modification would be needed to improve its drug features. Thus rather than perform more screening and characterization of new compounds (Task 2, c – e, of the original SOW), we decided to focus on doxorubicin, which has been well studied and is a FDA approved drug already used in prostate cancer. In fact, repurposing a known drug has many advantages in drug development, perhaps most importantly the speed with which one can get novel applications into the clinic. Our finding of selective actions of dox, specifically at low concentration, on AR target genes suggest novel uses in clinical application to prostate cancer, with hopes of reducing toxicity and resistance.

Described below is positive progress made in the second year (through 07/29/2016), focusing first on the mechanism by which dox enhances, or interferes with, AR action (Task 1e in the original SOW; Task 2c in the revised SOW). Since it seemed unlikely that dox was binding AR itself, but rather exerting its effect by differential intercalation into response element sequences, establishing mechanism is crucial. Thus we analyzed the protein-DNA interaction in greater detail to increase significance of data preliminarily presented in the first year (Fig. 2). Following investigation of the mechanism of dox action *in vitro*, we examined influence of dox on AR binding to target genes in chromatin by ChIP (Fig. 3). That dox enhances AR recruitment for sites where only one receptor monomer within the dimer can contact DNA is paradoxical but suggests the model presented in Fig. 4. The differential effect of low but not high dose dox on endogenous gene expression was investigated in greater detail (Fig. 5; Task 1b) to determine in what situations dox treatment may be clinically useful. This entailed analysis of dose and AR dependence for a broad array of AR target genes in varied cell lines. Global analysis (RNA-seq, ChIP-seq; Task 1c, d) is currently underway, and xenograft experiments (Task 3) will follow.

Differential effect of dox on AR binding to DNA response elements. Doxorubicin is one of the oldest and most effective chemotherapeutic agents used in cancer treatment. Though its mechanism of action is still not fully understood, dox is known to intercalate into DNA and disrupt topoisomerase II action, resulting in DNA double strand breaks (*2*). This activates the DNA damage response (DDR) system to repair the breaks; if repair is not successful, apoptosis is initiated. Recent studies indicate that AR regulates some genes involved in DDR, promoting prostate cancer radioresistance (*3*). This makes dox a plausible AR antagonist in prostate cancer. Our finding of a differential effect of dox on cARE vs. sARE activation led us to hypothesize that this was due to differential binding of AR to these elements in the presence of dox.

To test this, protein-DNA interaction was assessed by Electrophoretic Mobility Shift Assays (EMSA). Prostate cells gave complex results so nuclear extracts were prepared from HEK-293T cells transfected with AR. Extracts were reacted with ^{32}P-labeled cARE or sARE probes and increasing concentrations of dox (Fig. 2A). Shift 1 represents AR bound to DNA, since this complex only occurs with extracts containing AR and not with untransfected HEK293T cells.

The specificity of this complex was also previously confirmed using anti-AR antibody in supershift experiments. Although the same quantity of nuclear extract and probes were used in each reaction, the sARE complex formed in the absence of dox was less than the cARE complex, reflecting a 4-fold weaker affinity of AR for sAREs than cAREs, as previously noted (*4*). Shift 2 was also dependent on dox concentration but independent of AR, suggesting it was due to binding of other proteins or perhaps an artifact of probe migration.

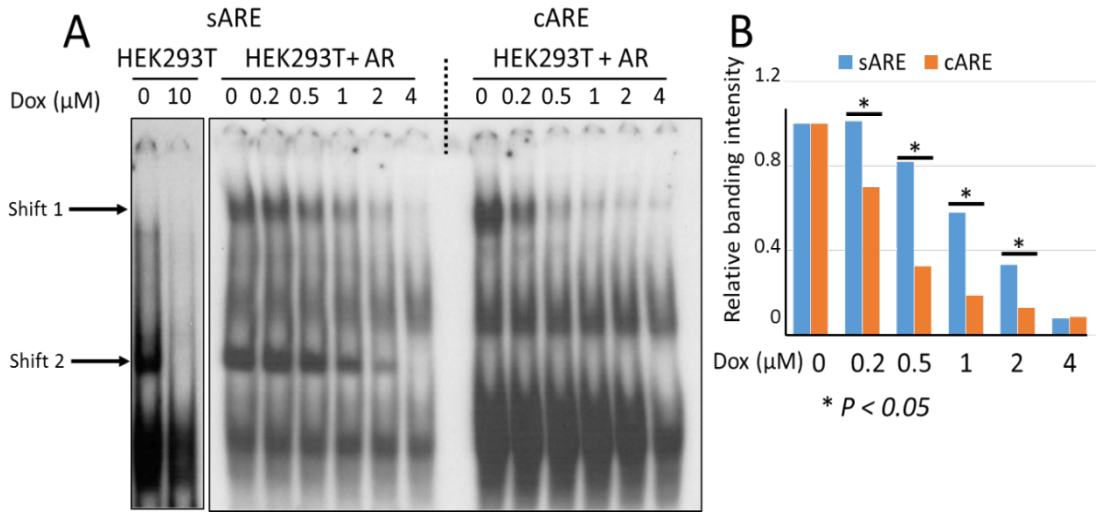

Fig. 2. Dox disrupts AR binding to cAREs more than to sAREs in EMSAs. HEK-293T cells were transiently transfected with AR plasmid for 48 hrs, treated with 10 nM R1881 for the last 12 hrs, and nuclear extracts prepared and stored at -80 C. Probes were synthesized by incubating 5 pmol sARE and cARE double-stranded oligonucleotides with 10 U T4 Polynucleotide Kinase and 2.5 μl of γ-^{32}PdATP (3000 Ci/mmol) for 60 min at 37 C, and free dATPs separated by sephadex column. For each reaction, 2 μg nuclear extract was mixed with 0.2 pmol ^{32}P-labeled probe and 50 ng/μl poly-dIdC as nonspecific competitor. The mixture was added into dox or DMSO and kept on ice 10 min before separation of complexes by electrophoresis. Gels were vacuum dried at 70 C and film exposed overnight at -20 C. A） Shift 1 is the specific AR-ARE complex and is disrupted by dox in a dose dependent manner, more readily for AR binding to cAREs than sAREs. Shift 2 is a complex formed by non-AR factors with sAREs. B) Developed film was scanned and the density of bands was analyzed using Image J software. This gel is representative of 3 independent EMSAs.

We used Image J to quantify the effect of dox on AR binding in EMSA (Fig. 2B). The intensity of the AR-cARE complex decreased more with increasing dox concentration than the AR-sARE complex, suggesting that AR-cARE binding is more sensitive to dox treatment. At higher concentrations, dox completely inhibited AR binding to both elements. It was paradoxical that the AR-cARE complex was more readily disrupted by dox than the sARE complex since there are two AR contact sites in the cARE vs. one in the sARE (see model in Fig. 4). This may reflect a distinct conformational change in DNA induced by dox that affects AR binding to repeats vs. half-sites. Binding of both monomers within the AR dimer to the cARE may be more sensitive to conformational changes, whereas binding to the sARE may be less stringent. It is also likely that intercalation of dox may have DNA sequence-specific structural effects that impact AR binding to one (sARE) or both (cARE) half-sites.

Effect of dox on AR recruitment to chromatin of AR target genes. To investigate whether dox interferes with AR recruitment to chromatin in cells as it does to AR binding to DNA *in vitro*, chromatin immunoprecipitation assays (ChIP) were performed following dox and hormone treatment of LNCaP cells. Most AR driven genes, such as PSA and TMPRSS2, have identifiable

cAREs in their promoters or enhancers, whereas some genes with more specific AR response have been noted to have nonconsensus elements (e.g., sAREs). SGK1 was noted to be AR-specific due to a nonconsensus sARE in its promoter (*5, 6*). A sARE element was also identified in the first intron of the SARG gene, which shows specific response to AR and not GR (*6*). Part of our overall hypothesis is that cARE-driven genes are pro-proliferative whereas sARE-driven genes are pro-differentiative or pro-survival. To examine the effect of dox on AR binding to endogenous genes, we isolated chromatin from dox-treated LNCaP cells, performed IP with AR or PolII antibodies, purified DNA precipitated by virtue of the bound protein and quantified specific sequences by qRT-PCR. The results from AR antibody ChIP assays clearly demonstrated that dox effectively prevented AR recruitment to cARE elements in the promoter/enhancers of the PSA and TMPRSS2 genes (Fig. 3A). In contrast, AR recruitment to sARE elements of SGK1 and SARG was stimulated by dox treatment (Fig. 3B). Similar trends as for AR were observed with Pol II antibody in ChIP assays, indicating the effect of dox on AR binding directly impacts gene expression, in both positive and negative directions. This has been substantiated for additional targets in additional cell lines.

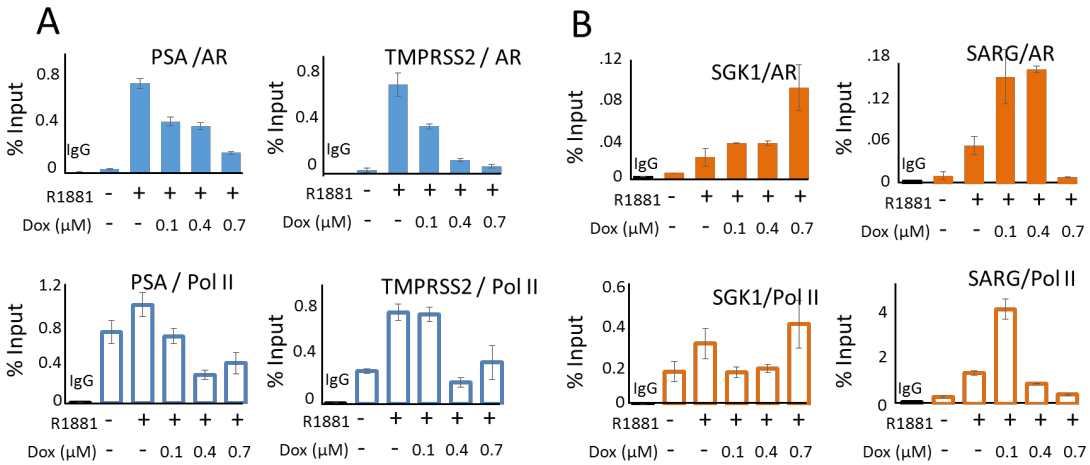

Fig. 3. Dox differentially affects AR recruitment to chromatin of AR target genes. LNCaP cells were cultured in RPMI 1640 media with 2% charcoal stripped serum for 48-72 hrs and then stimulated with 10 nM R1881 for 24 hrs. Cells were crosslinked with formaldehyde and lysed to harvest chromatin, which was then sonicated to 300-1000 bps on ice. ChIP assays were performed using AR antibody (N20, Santa Cruz Biotech), Pol II antibody (CTD4H8, Sata Cruz Biotech) and IgG. Purified DNA was quantified by quantitative real time PCR. A) qRT-PCR targeting promoter regions with cAREs of PSA and TMPRSS2 genes. B) qRT-PCR targeting sAREs in the promoter of SGK1 and intron 1 of SARG.

Based on EMSA and ChIP data, we propose a model of how dox differentially affects binding of AR dependent on response element (Fig. 4). Ligand-bound AR forms a dimer, each monomer of which contacts a half-site within the cARE whereas only one monomer binds a half-site in a sARE. AR-cARE binding is more sensitive to low concentrations of dox since intercalation into either half-site may cause conformational changes that disrupt the structure required for binding of both AR monomers. Such change is tolerated at low dox concentration for AR-sARE binding where AR needs to bind with only one monomer and the sequence conservation is less stringent. It is also likely that sequence differences between inverted repeat cAREs vs. half-site sAREs affect dox affinity to distinct elements and thus the affinity of AR binding. Additionally, AR binding to half-sites may be more dependent on neighboring bound proteins.

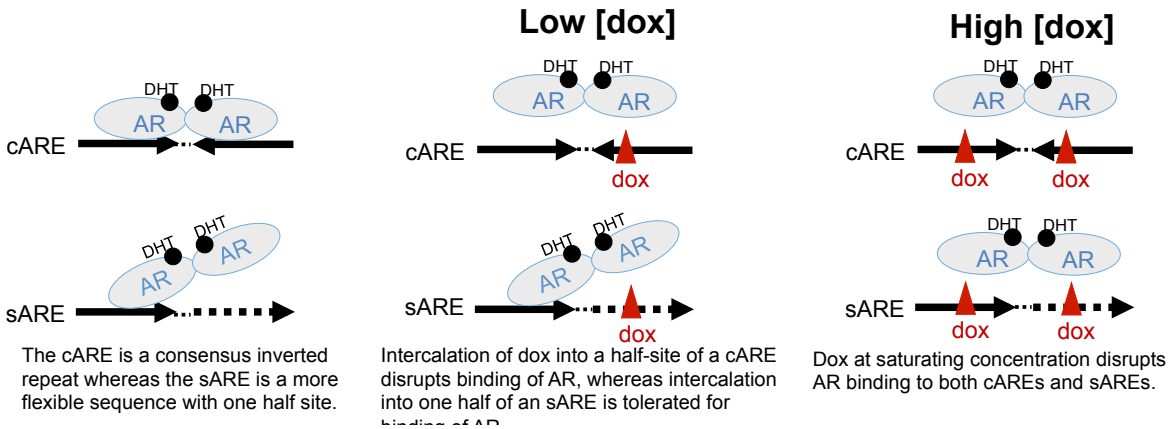

Fig. 4. A mechanism for differential effect of dox on sARE- vs. cARE-driven AR target genes.

The effect of dox on endogenous AR target gene expression. We next investigated whether effects of dox on AR recruitment to chromatin translated to the level of gene expression. Prior to the unbiased but expensive approach of RNA-seq, we sought additional examples of genes driven by sAREs, which are less well studied. We considered using commercially available PCR arrays of AR targets, or genes involved in DNA damage response, but few of these genes have characterized AREs. Therefore we sought candidate genes identified in the literature and deposited datasets (*5-10*). Several genes were chosen based on differential response to androgens and glucocorticoids, or cellular function, such as in DDR (*5, 7-9, 11*). Genes that showed greater than 2-fold response to R1881 in LNCaP cells were tested further for response to dox (Fig. 5).

The genes studied were all upregulated by hormone but showed distinct responses to dox. Furthermore, some intriguing gene interactions informative to cancer biology were revealed. Panel A includes genes that were upregulated by R1881 and efficiently repressed by dox, such as PSA, TMPRSS2, FKBP5 and NKX3.1, which are all classic cARE-driven AR target genes. This data is consistent with the AR-cARE binding data from ChIP (Fig. 3).

Panel B includes AR target genes induced by R1881 and further upregulated by dox. SARG is highly conserved between man and mouse, is repressed in most cancers (Oncomine), but its function remains unknown. SGK1 can be regulated by either AR or GR, dependent on context. High SGK1 expression in primary prostate cancer is associated with high AR and low GR levels; low SGK1 expression in androgen-deprived or untreated tumors is associated with poor prognosis (*12*). Like SARG, SGK1 expression is low in most tumors. Knock down of SGK1 inhibits AR-dependent LNCaP cell survival (*13, 14*). Therefore, a clinical benefit of SGK1 upregulation by dox may depend on tumor stage and AR/GR levels, and in some cases be useful in early stage disease or watchful waiting, and in other cases castration resistant prostate cancer (CRPC). Another gene found to respond similarly to SGK1 is NDRG1, a tumor suppressor involved in growth arrest, cell differentiation and prevention of metastasis (*15*). NDRG1 response to DHT more than Dex, supporting the idea that it is sARE-driven (*9*). Interestingly, NDRG1 is repressed by Myc, which was modestly down-regulated by dox, suggesting multiple pathways interact to regulate NDRG1 expression.

Panel C includes genes that were upregulated by R1881 but only modestly repressed by dox, declining to about half their androgen-induced level at 0.4 μM dox, a concentration causing complete repression of Panel A genes. While AR response elements of these genes have not been

mapped, ABCC4, TMEPAI and KLK4 respond more to DHT than Dex treatment (*9*). In another report, a DNA-binding polyamide targeting cAREs repressed expression of PSA, TMPRSS2 and FKBP5 but failed to repress HPGD and KLK4 (*11*). These results indicate some degree of androgen specificity for Panel C genes and suggest they may be driven by sAREs, other nonconsensus AREs, or be indirectly regulated by AR. Further investigation will clarify this.

In addition to these AR targets, we tested the effect of dox on a panel of genes involved in DNA damage response, e.g., POLE2, MAD2L1, FANCI, RFC3, POLA2, MCM7. These genes were reported to be AR-regulated through cARE elements (*3*). In our hands, 0.1 μM dox efficiently repressed their expression. However, R1881 treatment had little or no effect on these genes in LNCaP cells (not shown), thus it is not clear whether this repression is AR-dependent.

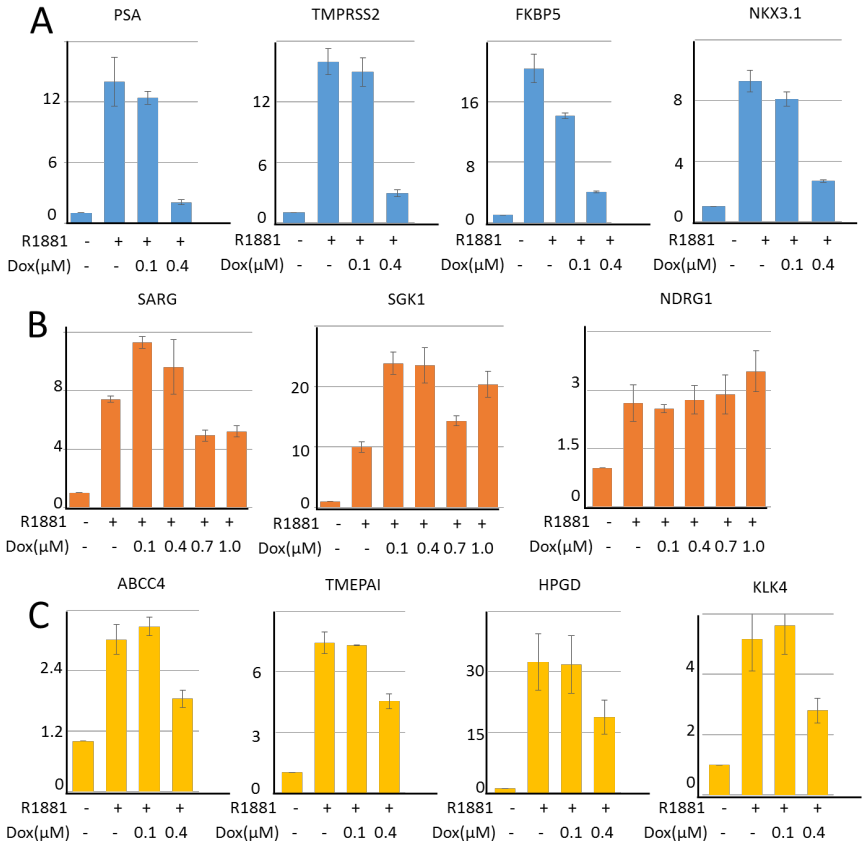

Fig. 5. Dox differentially regulates AR target gene expression. LNCaP cells were treated as in Fig. 3. RNA was extracted and qRT-PCR performed to quantify gene expression. A） Dox repressed cARE driven expression of PSA, TMPRSS2, FKBP5 and NKX3.1. B) Dox stimulated sARE driven gene expression of SARG, SGK1 and NDRG1. C) Dox partly repressed AR-induced expression of ABCC4, TMEPAI, HPGD and KLK4.

Dox treatment reveals an androgen-dependent tumor suppressor network. We previously noted that P21, a cell cycle regulator and tumor suppressor (*16*), was induced in LNCaP cells by dox but not by R1881. However, R1881 augmented dox induction, suggesting that AR may indirectly regulate P21. A candidate for the indirect regulator is the transcription factor KLF4, which activates P21 to cause cell cycle arrest (*17*). KLF4 is moderately upregulated by R1881 (1.7-fold) and further upregulated by dox (Fig. 6A), in an expression pattern similar to that of SARG and SGK1, suggesting that KLF4 may also be driven by a sARE-like AR binding site.

Also critical in P21 regulation is the classic tumor suppressor P53 (*18*). P53 and KLF4 binding sites overlap on the P21 promoter, leading to synergistic regulation (*17*). While P53 is not generally stimulated by androgen, ARE-like sequences have been noted (*19*), one of which occurs in the first intron and matches the sARE defined by Sahu et al. (*1*). By ChIP, we showed that AR localization to this element was stimulated by R1881 and further increased by dox (Fig. 6B). In contrast, Pol II localization to this element was decreased by R1881 and dox, consistent with decreased P53 expression (Fig. 6B). This suggests that AR negatively regulates P53 via the sARE in the first intron. That dox promotes AR localization but does not increase repression may be because the resulting DNA damage response upregulates P53 expression by additional means

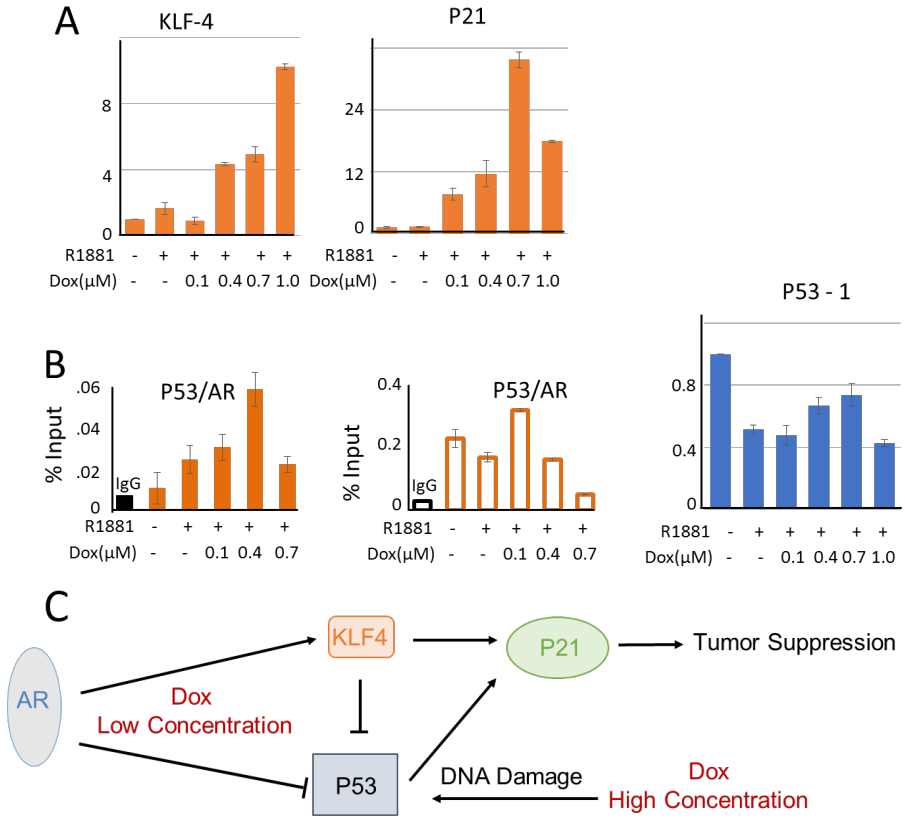

Fig. 6. Dox affects KLF4, P21 and P53 gene expression. LNCaP cells were grown and treated as before (see Fig. 5 legend), followed by RNA or ChIP assays. A) qRT-PCR assays show KLF4 expression is upregulated by R1881 and further stimulated by dox, whereas P21 expression is upregulated only by dox. B) AR recruitment to the sARE region in intron 1 of the P53 gene is stimulated by both R1881 and dox (left), but P53 expression was repressed, as indicated by PolII binding (middle) and RNA level (right). C) Dox shifts the KLF4, P53 and P21 axis toward PCa suppression through both AR-sARE and AR-independent pathways (see text).

to override AR-driven P53 repression. Interestingly, in breast cancer KLF4 directly represses P53 transcription, switching from a cell-cycle inhibitor into an oncoprotein following P21 loss (*20*). Moreover, in prostate cancer, P53 negatively regulates AR when over-expressed (*21*). These finding support the idea that AR and P53 regulate each other at multiple levels (*19*). The model of Fig. 6C depicts that AR directly upregulates KLF4 and downregulates P53 through sAREs. KLF4 suppresses tumor progression by promoting P21 and inhibiting P53 expression; shifts in balance can switch KLF4 to oncogenesis. Finally, higher dox concentration triggers DNA damage response and increased P53 and P21 expression, regardless of AR action (Fig. 6C).

Summary. We have shown that dox inhibits AR-dependent gene expression from consensus inverted repeats (cAREs) to a greater extent than from AR-selective response elements (sAREs). This likely depends on conformational changes caused by dox intercalation into DNA that disrupts binding of the AR dimer to a cARE more than to a sARE. The greater stability of the AR-sARE complex is also likely enhanced by interaction with additional factors, both in nuclear extracts and *in vivo*, underlying greater recruitment of AR into chromatin and enhanced transcription of sARE-driven AR targets in response to low dox. Gene expression patterns in response to dox in LNCaP cells supports our hypothesis that selective AR modulation can distinguish proliferative from differentiative gene promoters, ultimately altering cell growth characteristics. An intriguing example is the KLF4 / P53 / P21 axis that demonstrates AR regulation and dox effects at multiple levels, and demonstrates how changing levels of a few key proteins can switch cells from differentiative to proliferative growth. In total, our data demonstrate that low dose dox treatment shifts prostate cancer cells toward differentiation and growth arrest. This will be pursued *in vivo* to determine whether combined low dox, low antiandrogen treatment leads to more differentiated cancer cell growth, and ultimately fewer side effects of therapy and less pressure to develop therapy resistance.

KEY RESEARCH ACCOMPLISHMENTS:

- EMSA assays demonstrated that dox affects AR binding to consensus vs. selective elements (cARE, sARE) differentially. A model is proposed to explain the mechanism.

- ChIP assays confirmed that low dose dox increases AR recruitment to sAREs and decreases AR recruitment to cAREs.

- Gene expression analysis confirmed that cARE-driven genes are efficiently repressed by dox, whereas sARE-driven genes are upregulated or only partly repressed by dox.

- Accumulated data, and characterization of the KLF4/P21/P53 regulatory network, support that dox treatment at low concentrations upregulates genes associated with differentiation or growth arrest.

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Opportunities for Training and Professional Development
Dr. Shihan He began on this project as a postdoctoral fellow and has recently been appointed a Research Specialist. He has been responsible for the majority of studies reported here, under my mentorship. He wrote the draft of this progress report, gaining writing experience and learning from my editing. He has presented this work at a departmental poster session within the University, and also at this year's Multi-Institutional Prostate Cancer Program Retreat (March 13-15, 2016, Fort Lauderdale, FL). This annual meeting is largely attended by investigators from the Prostate SPORES of Harvard, Fred Hutchinson, Hopkins, Sloan-Kettering and University of Michigan, and is an excellent opportunity for junior investigators to interact with more established researchers in a relaxed environment.

Dissemination of Results
The abstract for the 2016 Multi-Institutional Prostate Program Retreat is appended.

Next Reporting Period
We will continue analysis of differential effects of dox on gene expression, fojr global genomic analyses and in mouse xenograft experiments, as planned. We will also continue to probe the molecular mechanism by which dox elicits these differential effects in AR gaction.

4. IMPACT
There is nothing to report during this period but we expect to be preparing a manuscript soon.

5. CHANGES/PROBLEMS
We have performed the expanded screen of Task 2 but as no promising candidates were found we will not pursue the rest of that Task but instead focus on doxorubicin, which was identified in the pilot screen and remarkably validates our hypothesis of distinguishing sets of AR target genes by their promoter sequences. We have made progress but not as rapidly as hoped, in part due to an accident that kept me out of the lab for 2 months, followed by slow recovery. We have revised our SOW (attached) going forward to take this into account.

6. PRODUCTS
There is nothing significant to report during this period. The abstract from the Prostate Retreat mentioned above is appended.

7. PARTICIPANTS

Name:	Diane M. Robins, Ph.D.
Project Role:	P.I.
Research ID (ORCID):	0000-0001-6727-6309
Person Mo.:	1
Contribution:	Dr. Robins conceives and oversees the experiments and reports the results
Funding:	10% from this award, 25% from an NCI-RO1 (until 02/28/16), 2.5% from the SPORE

Name:	Michele Brogley
Project Role:	Res. Assistant

Research ID: n/a
Person Mo.: 5
Contribution: Ms. Brogley assists with cell culture, molecular assays and lab managing
Funding: Ms. Brogley's effort on this project has increased to 40%

Name: Shihan He, Ph.D.
Project Role: Unchanged

The funding of the PI decreased when her RO1 ended 02/28/16.

8. SPECIAL REPORTING REQUIREMENTS – N/A

9. APPENDICES:

- abstract from Multi-Institutional Prostate Retreat attached
- Revised Statement of Work, submitted 01/06/2017, attached

9th ANNUAL MULTI-INSTITUTIONAL PROSTATE CANCER PROGRAM RETREAT

March 13-15, 2016
W Hotel Fort Lauderdale
Fort Lauderdale, Florida

ABSTRACT SUBMISSION FORM

Due Date: December 4, 2015

Please Note: These abstracts are to be submitted for posters with the knowledge that some may be chosen for a talk or poster presentation.

First Name: Shihan

Last Name: He

Check one of the following boxes:

Organization:

- ☐ Dana-Farber Harvard Cancer Center
- ☐ Fred Hutchinson Cancer Research Center
- ☐ Johns Hopkins Medical Institutions
- ☐ Memorial Sloan-Kettering Cancer Center
- X University of Michigan Comprehensive Cancer Center

PI of Your Research Program/Lab: Diane Robins

Telephone: 734-764-4563

Email: shihanh@med.umich.edu

ABSTRACT

Your complete abstract should be no longer than 3,000 characters (450–500 words).

Insert text following the colon after each heading

Title: A high-throughput screen for selective AR regulators reveals a novel role for doxorubicin

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Complete Author Listing:

Shihan He, Pia Bagamasbad, Diane M. Robins

Body:

Prostate cancer (PCa) depends on androgen receptor (AR) activity for its development and progression. Androgen deprivation therapy initially slows the disease but ultimately leads to resistance. In castration resistant prostate cancer (CRPC), constitutively active ARs, such as those lacking the ligand binding domain, are often found. This highlights a need to develop new anti-AR therapeutic strategies. Our goal is to selectively block expression of AR target genes involved in tumor proliferation while retaining expression of genes for differentiation. AR binds to two types of androgen response elements, the consensus androgen response element (cARE), which is an inverted repeat recognized by other steroid receptors, and a more selective androgen response element (sARE), which conforms to a direct repeat or half-site. Some data supports the notion that cAREs drive genes promoting cell growth while sAREs are associated with genes regulating differentiation. Thus compounds may selectively interfere with AR action by differential interactions with AR, response elements or other transcriptional components.

To identify selective AR modulators, we developed a high-throughput promoter-dependent screen using transfected fluorescent reporters driven by multimerized cAREs or sAREs. More than 10,000 compounds from several libraries, including 2,000 FDA-approved drugs, were screened. One hit, doxorubicin (dox), selectively inhibited cARE-driven fluorescence and was further validated in luciferase assays in prostate cells. Dox is an intriguing hit since it is commonly used in cancer chemotherapy, intercalates into DNA and initiates DNA damage response (DDR). AR also plays a role in DDR. We hypothesized that preferential interaction of dox with different DNA sequences could selectively interfere with AR binding and thus differentially affect AR target gene activation. In LNCaP cells and RWPE cells stably expressing AR, dox treatment repressed AR targets FKBP5, KLK1, NKX3 and TMPRSS2, but increased expression of Aq3, SGK-1 and tumor suppressor P21, which are more associated with differentiation. To test an effect on DNA binding, ^{32}P-labeled cARE or sARE probes were interacted with dox and nuclear extracts from AR-expressing cells. Electrophoretic Mobility Shift Assays (EMSA) confirmed that AR-cARE binding was more sensitive to dox than AR-sARE binding. We further showed selective effects of dox in a chromatin context: ChIP assays in LNCaP and LAPC-4 cells showed that dox promoted AR binding to P21 and SGK1 promoters. This data provides proof of concept that compounds can differentially regulate AR target gene expression via selective interaction with distinct promoter elements. Further, this leads to the suggestion that combined therapy with low dose dox and antiandrogens may prove to be a more selective and less toxic treatment for some cases of PCa.

Acknowledgments/Funding:

Support was from the DOD to DMR (W81XWH-14-1-0292) and to PB (W81XWH-11-1-0360).

Selective AR Modulators of Proliferative vs. Differentiative Gene Promoters
Award W81XWH-14-1-0292

STATEMENT OF WORK – revised 12/30/2016
Project started 07/30/2014

Specific Aim 1: To perform a high-throughput screen for selective modulators of AR gene regulation	
Major Task 1: Perform a larger screen for novel compounds using an improved protocol	months
Subtask 1: Scale up the pilot assay and perform larger screen optimized for transfection and sensitivity. Cell lines used: HeLa-AR [from Elizabeth Wilson, UNC]	1-4
Subtask 2: Re-assay primary hits for dose response; re-test fresh powder samples. Cell lines used: HeLa-AR [from Elizabeth Wilson, UNC]	5-7
Subtask 3: Prioritize hits using MScreen data assessment and guidance from the Center for Chemical Genomics	8-9
Subtask 4: Validate hits for effects on transfected cARE- or sARE-driven reporters, with and without AR and hormone. Cell lines used: CV-1, RWPE-1, PC-3, LNCaP [all these lines are from ATCC]	9-12
Milestone(s) Achieved: identification of doxorubicin as a major hit that validated robustly, thus becoming the focus of our ongoing effort	12
Specific Aim 2: Validate doxorubicin modulation of endogenous AR target genes and probe mechanism of action in cells	
Major Task 2: Probe modulatory effect of doxorubicin on AR control and cell phenotype	
Subtask 1: Dox effect on proliferative vs. differentiative gene expression will be tested in benign vs. malignant, AR+ vs. AR- prostate cells, at the RNA level by qPCR Cell lines additional to those in Aim 1 above: LAPC4 [C. Sawyers, MSKCC], C4-2B [Evan Keller, UM]	5-18
Subtask 2: Effects on proliferation, apoptosis, senescence and invasion will be determined in prostate cells representing early vs. late cancer Cell lines used: LNCaP [ATCC], C4-2B [Evan Keller, UM]	9-26
Milestone(s) Achieved: characterization of the AR-selective and biological effects of dox	16
Major Task 3: Examine mechanism of selective effect of doxorubicin on AR action	
Subtask 1: Examine whether dox selectivity works via AR binding to DNA, by in vitro (EMSA) assays and effect on recruitment of AR to chromatin of select genes	12-18

Subtask 2: Analyze global gene expression by RNA-seq for a broad unbiased view	22-28
Subtask 3: Identify global effects on AR recruitment to chromatin by ChIP-seq	24-30
Milestone(s) Achieved: determine how doxorubicin attains selective modulation of AR regulation	30
Specific Aim 3: Test antitumor efficacy of selective modulators on mouse tumors in vivo	
Major Task 4: Determine differential antitumor effects of low vs high doses of doxorubicin in mice	
Subtask 1: LAPC4 cell tumors will be established and grown subcutaneously in mice [10 mice per group x 9 groups = 90 mice total]	24-36
Subtask 2: Differential effects on metastasis, if noted for LAPC4 tumors, will be assessed in C4-2B cell tumors by real time bioimaging. [10 mice per group x 2 groups = 20 mice total]	32-36
Subtask 3: Histopathology and molecular characterization of tumors to analyze selective effects of doxorubicin on AR regulation	30-36
Milestone(s) Achieved: Characterization of effects of low dose doxorubicin on AR-dependent tumor growth in vivo; publication of 1-2 peer reviewed papers	36